Isolation and full-genome sequences of Japanese encephalitis virus genotype I strains from Cambodian human patients, mosquitoes and pigs

Veasna Duong,1 Rithy Choeung,1 Christopher Gorman,1 Denis Laurent,2 Yoann Crabol,3 Channa Mey,1 Borin Peng,1 Juliette Di Franceso,4 Vibol Hul,1 Heng Sotthi,2 Ky Santy,2 Beat Richner,2 Jean-David Pommier,3 San Sorn,4 Véronique Chevalier,3,5 Philippe Buchy,6 Xavier de Lamballerie,7,8 Julien Cappelle,3,5,9 Paul Francis Horwood1,10 and Philippe Dussart1,*

Abstract

Japanese encephalitis remains the most important cause of viral encephalitis in humans in several southeast Asian countries, including Cambodia, causing at least 65 000 cases of encephalitis per year. This vector-borne viral zoonosis – caused by Japanese encephalitis virus (JEV) – is considered to be a rural disease and is transmitted by mosquitoes, with birds and pigs being the natural reservoirs, while humans are accidental hosts. In this study we report the first two JEV isolations in Cambodia from human encephalitis cases from two studies on the aetiology of central nervous system disease, conducted at the two major paediatric hospitals in the country. We also report JEV isolation from Culex tritaeniorhynchus mosquitoes and from pig samples collected in two farms, located in peri-urban and rural areas. Out of 11 reverse-transcription polymerase chain reaction-positive original samples, we generated full-genome sequences from 5 JEV isolates. Five additional partial sequences of the JEV NS3 gene from viruses detected in five pigs and one complete coding sequence of the envelope gene of a strain identified in a pig were generated. Phylogenetic analyses revealed that JEV detected in Cambodia belonged to genotype I and clustered in two clades: genotype I-a, mainly comprising strains from Thailand, and genotype I-b, comprising strains from Vietnam that dispersed northwards to China. Finally, in this study, we provide proof that the sequenced JEV strains circulate between pigs, Culex tritaeniorhynchus and humans in the Phnom Penh vicinity.

INTRODUCTION

Japanese encephalitis (JE) is the most important cause of epidemic encephalitis worldwide. The disease is endemic in many southeast Asian countries, where it causes at least 65 000 cases of encephalitis per year [1]. However, this figure is likely to be a gross underestimate, as surveillance and reporting systems are not well developed in the region [2]. Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus transmitted primarily by Culex mosquitoes to ardeid wading birds and pigs. JE was described in Japan from the 1870s onwards, and the prototype Nakayama strain was isolated from a fatal case in 1935. Since then, the disease apparently spread across Asia to affect most of China and the Asian subcontinent, all of southeast Asia, and the Pacific Rim, reaching northern Australia. Previous studies indicate that JEV can be classified into five genotypes (I to V), based on the diversity of the nucleotide
sequences of the E protein gene or the gene encoding the complete polyprotein, with most isolates classified as genotype I (GI) or genotype III (GIII) [3]. With more availability of JEV sequences, a recent phylogenetic study of E protein gene sequences revealed that genotype I can be subdivided into two clades, GI-a and GI-b, with the majority of isolates clustering in GI-b. GI-a mainly comprises Thailand strains from the late 1990s and early 2000s [4].

Humans are a dead-end host for JEV, as the resulting viraemia is not high enough to reinfect mosquitoes [5]. Although less than 1% of humans infected with JEV will develop JE, the mortality rate is very high in children and adolescents [7–11]. JE is recognized as the most prevalent cause of encephalitis and febrile illness in Cambodia, particularly in children and adolescents [7–11]. Although numerous encephalitis aetiology studies have confirmed the importance of this virus, no information on the genotypes of circulating strains has been reported in Cambodia since the isolation of JEV genotype I from a mosquito in 1967 [12]. In this study we report the first two isolations and full-genome sequences of two strains from two human encephalitis cases of JEV from Cambodia, and make a comparison with recent isolates from mosquitoes and pigs.

RESULTS

Human samples

Among 1160 paediatric AME cases, 35.0% (n=406) resulted in confirmed or highly probably aetiologies. JEV was the most commonly identified pathogen (n=283, 24.4%): 20.3% (n=236) of cases were categorized as ‘confirmed’ (n=3) or ‘highly probable’ (n=233), as defined by the detection of JEV RNA or IgM antibodies in the cerebrospinal fluid (CSF), respectively. Further, 4.1% (n=47) of cases were categorized as ‘suspected’, as defined by IgM seroconversion from paired sera [11]. From the three confirmed JEV patients, one JEV strain detected in 2013 was successfully isolated from CSF on C6/36 cells. The second human strain was identified from 1 patient in CSF by real-time reverse-transcription polymerase chain reaction (RT-PCR) in December 2015, from among the 147 patients included in the southeast Asia encephalitis project (SE Ae, www.seaeproject.org), and it was successfully isolated on C6/36 cells. Full-genome sequences were obtained from both JEV isolates (Table 1).

Pig samples

From the 4 pig cohorts (April 2014 to October 2015), which each included 15 pigs, we detected JEV by real-time RT-PCR in 8 pig blood samples (Table 1). Two were from 2014 and six were from 2015. All sera were inoculated into C6/36, and two supernatants from 2015 were found to be positive, with full genomes being obtained. Full-genome sequencing attempts on the other six blood samples were unsuccessful. However, partial sequences of NS3 or envelope genes were generated from the original samples (Table 1).

Mosquito samples

A total of 11,037 mosquitoes were collected from the peri-urban farm and 6 mosquito genera were identified, including Aedes, Armigeres, Anopheles, Culex, Mansonia and Mimomyia (Table 2). Culex tritaeniorhynchus was the most abundant species, followed by Cx. gelidus and the Cx. vishnui subgroup. A large majority of the captured mosquitoes were female (99%). A total of 1175 pools were constituted by their week of collection, genera, species and sex, and tested for JEV by real-time RT-PCR. One pool of 10 Cx. tritaeniorhynchus females captured in September 2014 was found to be positive. JEV was subsequently successfully isolated from this pool after inoculation onto C6/36 cells. The full-genome sequence was obtained from this JEV isolate (Table 1). The minimum infection rate (MIR) in the mosquitoes – calculated as the number of positive pools divided by the total number of mosquitoes – was 0.09/1000 Cx. tritaeniorhynchus.

Sequencing and phylogenetic analysis

Among the five JEV strains successfully isolated in C6/36 cells, we were able to generate the full virus genome sequences from two human samples, two pig samples (one from a peri-urban farm and one from a rural farm) and one mosquito pool. Five additional partial sequences of the NS3 gene from five pigs and one complete coding sequence of the envelope (E) gene from one pig were generated (Table 1). We were not able to sequence the genome of a virus detected in one pig in 2014.

Phylogenetic analysis was conducted with 5 full genome sequences, 10 partial sequences of NS3 and 6 complete coding sequences of the E gene from Cambodian viruses with reference to JEV sequences belonging to the 5 genotypes available in GenBank. The accession numbers of all the obtained sequences are presented in Figs 1, 2 and S1. The phylogenetic trees for the full genome and partial and complete coding of the E gene sequences show similar phylogenetic similarity to those of Cambodian JEV strains belonging to genotype I, clustered in two clades, GI-a and GI-b (Figs 1, 2 and Fig. S1, available with the online Supplementary Material). Subsequently, trees constructed with partial NS3 sequences (five JEV isolates and five additional sequences generated by direct sequencing on original specimens; Fig. S1) and complete coding sequences of the E gene (five JEV isolates and one additional sequence generated by direct sequencing on original specimens; Fig. 2) were analysed.

The phylogenetic tree for the partial NS3 sequences was constructed with 2 JEV strains from humans, 1 from Cx. tritaeniorhynchus, 7 from pigs and 36 sequences from GenBank. The two human strains clustered in both the GI-a and GI-b groups. The human strain isolated in 2015 (GenBank accession no. KY927816) clustered together with Cambodian strains from mosquitoes and pigs collected in 2014 within GI-a (Fig. S1). The human strain isolated in 2014 within GI-a (Fig. S1). The human strain isolated in 2014 within GI-a (Fig. S1). The human strain isolated in 2014 within GI-a (Fig. S1).
### Table 1. Description of JEV-positive specimens collected from humans, pigs and mosquitoes

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Host</th>
<th>Age</th>
<th>Sex</th>
<th>Project</th>
<th>Type of sample</th>
<th>Origin</th>
<th>Sampling date</th>
<th>Real-time RT-PCR (Ct value)</th>
<th>Conventional RT-PCR (NS3)</th>
<th>Virus isolation</th>
<th>Sequence (GenBank accession no.)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME0802</td>
<td>Human</td>
<td>3 years</td>
<td>Female</td>
<td>AME</td>
<td>CSF</td>
<td>Ratanakiri province</td>
<td>19 May 2013</td>
<td>Pos (40.00)</td>
<td>Pos</td>
<td>Pos</td>
<td>Full genome (KY927819)</td>
<td>GI-b</td>
</tr>
<tr>
<td>C081</td>
<td>Human</td>
<td>8 years</td>
<td>Male</td>
<td>SEAe</td>
<td>CSF</td>
<td>Prey Veng province</td>
<td>7 July 2015</td>
<td>Pos (39.80)</td>
<td>Pos</td>
<td>Pos</td>
<td>Full genome (KY927816)</td>
<td>GI-a</td>
</tr>
<tr>
<td>A14 B8</td>
<td>Pig</td>
<td>137 days</td>
<td>NA</td>
<td>SEAe</td>
<td>Serum</td>
<td>Ta Khmau city, Kandal province</td>
<td>27 June 2014</td>
<td>Pos (36.00)</td>
<td>Neg</td>
<td>Neg</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>B14-B4</td>
<td>Pig</td>
<td>85 days</td>
<td>Male</td>
<td>SEAe</td>
<td>Serum</td>
<td>Ta Khmau city, Kandal province</td>
<td>29 September 2014</td>
<td>Pos (32.00)</td>
<td>Pos</td>
<td>Neg</td>
<td>Partial NS3 (KY927809)</td>
<td>GI-a</td>
</tr>
<tr>
<td>C04-B6</td>
<td>Pig</td>
<td>117 days</td>
<td>Male</td>
<td>ComAcross</td>
<td>Serum</td>
<td>Ta Khmau city, Kandal province</td>
<td>10 August 2015</td>
<td>Pos (33.70)</td>
<td>Pos</td>
<td>Neg</td>
<td>Partial NS3 (KY927813)</td>
<td>GI-b</td>
</tr>
<tr>
<td>C12-B1</td>
<td>Pig</td>
<td>65 days</td>
<td>Male</td>
<td>ComAcross</td>
<td>Serum</td>
<td>Ta Khmau city, Kandal province</td>
<td>19 June 2015</td>
<td>Pos (33.84)</td>
<td>Pos</td>
<td>Neg</td>
<td>Partial NS3 (KY927810)</td>
<td>GI-b</td>
</tr>
<tr>
<td>C14-B3</td>
<td>Pig</td>
<td>86 days</td>
<td>Male</td>
<td>ComAcross</td>
<td>Serum</td>
<td>Ta Khmau city, Kandal province</td>
<td>10 July 2015</td>
<td>Pos (33.39)</td>
<td>Pos</td>
<td>Pos</td>
<td>Full genome (KY927817)</td>
<td>GI-b</td>
</tr>
<tr>
<td>D03-B9</td>
<td>Pig</td>
<td>145 days</td>
<td>Female</td>
<td>ComAcross</td>
<td>Serum</td>
<td>Rural area, Kandal province</td>
<td>9 September 2015</td>
<td>Pos (29.60)</td>
<td>Pos</td>
<td>Pos</td>
<td>Full genome (KY927818)</td>
<td>GI-b</td>
</tr>
<tr>
<td>D08-B9</td>
<td>Pig</td>
<td>145 days</td>
<td>Female</td>
<td>ComAcross</td>
<td>Serum</td>
<td>Rural area, Kandal province</td>
<td>9 September 2015</td>
<td>Pos (29.53)</td>
<td>Pos</td>
<td>Neg</td>
<td>Partial NS3 (KY927811)</td>
<td>GI-b</td>
</tr>
<tr>
<td>D15-B12</td>
<td>Pig</td>
<td>174 days</td>
<td>Male</td>
<td>ComAcross</td>
<td>Serum</td>
<td>Rural area, Kandal province</td>
<td>8 October 2015</td>
<td>Pos (29.26)</td>
<td>Pos</td>
<td>Neg</td>
<td>Partial NS3 (KY927812)</td>
<td>GI-b</td>
</tr>
<tr>
<td>639A37Cxtri</td>
<td>Culex</td>
<td>NA</td>
<td>Female</td>
<td>CamAcross</td>
<td>Mosquito</td>
<td>Ta Khmau city, Kandal province</td>
<td>12 September 2014</td>
<td>Pos (20.10)</td>
<td>Pos</td>
<td>Pos</td>
<td>Full genome (KY927815)</td>
<td>GI-a</td>
</tr>
</tbody>
</table>

NA: not available.
2013 (accession no. KY927819) belonged to the GI-b clade and did not group with the other seven Cambodian pig strains detected in 2015, but with a strain isolated in Laos in 2009. All of the JEV strains isolated and/or detected from pigs (n=7) were from Kandal province in peri-urban (n=4) and rural (n=3) areas and belonged to the GI-a and GI-b clades (Table 1). There was no association between the clade and the location of the pig farm. However, all of the JEV strains from 2015 belonged to clade GI-b.

Due to limited availability of NS3 gene reference sequences, a larger dataset of envelope sequences comprising 131 sequences was analysed. The phylogenetic tree of E gene sequences showed that all six Cambodian strains (two sequences from humans, one from a mosquito pool and three from pigs) were divided into three lineages (Fig. 2). Lineage 1 consisted of the strain detected in mosquitoes (accession no. KY927815) isolated in Kandal province in 2014 and the strain of human origin (accession no. KY927816) from Prey Veng province isolated in 2015, which both clustered closely with Thai strains isolated in 2004–2005 in clade GI-a. The percentage of nucleotide identity based on the complete coding E sequence between the Cambodian and Thai strains was 97.2–98.3% (Table S2). Lineage 2 consisted of three strains obtained from pigs (accession nos KY927811, KY927812 and KY9278158) detected from Kandal province (southern part) in 2015, which grouped closely with the Vietnamese strains isolated in 2005. The percentage of nucleotide identity between the Cambodian and Vietnamese strains varied from 95 to 96% (Table S2). Lineage 3 consisted of a human strain (accession no. KY927819) isolated in 2013 from Ratanakiri province (located at the border with Laos), which grouped closely with a Lao strain from 2009, within the same cluster of other strains isolated in China in 2005, in Japan in 2009 and in Vietnam in 2006. The percentage of nucleotide identity between the Cambodian strain and the Lao strain was 99.5%, while it was 99.0% for the Chinese and Japanese strains, and 98.8% for the Vietnamese strain (Table S2). The percentage of nucleotide identity between the Cambodian strains varied from 98.1 to 98.9% within the same lineage, and from 91.4 to 95.6% between lineages (Table S2).

**DISCUSSION**

JEV is still a major health problem in southeast Asia and is the most frequent aetiology of meningoencephalitis in Cambodia [11]. In this study, we report the detection of JEV in humans, pigs and mosquitoes, and provide the first full-genome sequences of JEV in Cambodia since the first report in 1967. All of the Cambodian JEV strains belonged to genotype I and clustered in GI-a and GI-b. Previous reports indicated that GI-a diverged in Thailand or Cambodia, whereas GI-b diverged in Vietnam and then dispersed northwards to China and subsequently to Japan, Korea, and Taiwan [4].

GIII is among the most frequently detected genotype along with GI in Asia and was frequently isolated in Asia until the 1990s [13]. Recent studies have demonstrated that GI strains have emerged and are gradually replacing GIII in many countries across Asia, including China [14], Thailand [15], Korea [16],

Table 2. List of mosquito species collected and results of JEV testing

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Sex</th>
<th>No. of mosquitoes (N=11037)</th>
<th>Percentage</th>
<th>No. of pools (n=1175)</th>
<th>Real-time RT-PCR</th>
<th>Conventional RT-PCR</th>
<th>Virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes aegypti</em></td>
<td>F</td>
<td>3</td>
<td>0.03 %</td>
<td>3</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1</td>
<td>0.01 %</td>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td><em>Armigeres spp.</em></td>
<td>F</td>
<td>5</td>
<td>0.05 %</td>
<td>3</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>5</td>
<td>0.05 %</td>
<td>2</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td><em>Anopheles spp.</em></td>
<td>F</td>
<td>14</td>
<td>0.13 %</td>
<td>4</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td><em>Culex quinquefasciatus</em></td>
<td>F</td>
<td>686</td>
<td>6.22 %</td>
<td>85</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>90</td>
<td>0.82 %</td>
<td>15</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td><em>Cx. gelidus</em></td>
<td>F</td>
<td>1593</td>
<td>14.43 %</td>
<td>167</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>8</td>
<td>0.07 %</td>
<td>4</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td><em>Cx. tritaeniorhynchus</em></td>
<td>F</td>
<td>7218</td>
<td>65.40 %</td>
<td>729</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>5</td>
<td>0.05 %</td>
<td>2</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td><em>Cx. vishnui subgroup</em></td>
<td>F</td>
<td>1387</td>
<td>12.57 %</td>
<td>147</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>6</td>
<td>0.05 %</td>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td><em>Cx. fuscoccephala</em></td>
<td>F</td>
<td>2</td>
<td>0.02 %</td>
<td>2</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td><em>Mansonia sp</em></td>
<td>F</td>
<td>12</td>
<td>0.11 %</td>
<td>8</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1</td>
<td>0.01 %</td>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
<tr>
<td><em>Mimomyia sp</em></td>
<td>F</td>
<td>1</td>
<td>0.01 %</td>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not available.
Japan [17], Malaysia [18], Vietnam [19], India [20] and Taiwan [21]. We did not detect any GIII strains in Cambodia, although it is still reported to be co-circulating with GI in Vietnam [22] and China [14]. This could potentially be explained by the small number of isolates we obtained from humans and mosquitoes. We detected eight JEV strains in pigs over 2 years from two locations separated by 30 km (Ta Khmau and Kandal). Interestingly, the strains belonging to GI-b circulating in 2015 in both the peri-urban farm and the rural farm (n=6) were not related to the strain circulating in pigs in 2014 in the same peri-urban farm, which belonged to GI-a, but the significance of this observation is probably limited, given the limited number of strains included in the study. The higher detection rate for GI-b compared to GI-a could be explained by the fact that GI-b viruses have greater multiplicative ability than GI-a, with higher virus titres [4].

Phylogenetic analysis of the E gene showed that six sequences of Cambodian JEV strains clearly grouped into three lineages clustered close to the Thai strains (lineage 1: sequences KY927815 and KY927816), the Vietnamese strains (lineage 2: sequences KY927814, KY927817 and KY927818) and a Lao strain (lineage 3: sequence KY927819). The second and third lineages clustering within GI-b can be explained by the geographical location of the detected JEV strains. Furthermore, GI-a mainly consists of strains detected in Thailand and Cambodia, with one strain from Australia. None of the available Vietnamese JEV strains clustered in GI-a, with the exception of one strain (JN574432) detected in a mosquito in 2005, which had 94.2 % homology with the Cambodian strains, which is lower than that for the GI-a Thai strains (>97%). Moreover, although the mosquito and human strains in lineage I within GI-a were detected in two southern Cambodian provinces close to Vietnam, we could not find any evidence of lineage I circulation in southern Vietnam.
The detection of JEV in pigs and mosquitoes around pig farms located in both urban and peri-urban areas confirmed that JEV is not only a rural disease, and indicated that there is an increased risk of JEV infection in areas with pig farming [23]. Interestingly, within GI-a, the JEV strains detected in an encephalitis patient, in mosquitoes and in pigs grouped closely, demonstrating the cycle of JEV infection.

From the trap placed close to pig farms, Cx. tritaeniorhynchus, the main competent vector for JEV, was captured at a significantly higher rate (65.4%) than the other mosquito species in our study. In Vietnam, pigs were associated with increased numbers of Cx. tritaeniorhynchus [22]. The increased number of competent JEV vectors around pig farms and the high circulation of JEV in pigs indicate the increased risks associated with pigs in areas where there is animal farming, regardless of whether the setting is urban or rural.

The MIR of 0.091/1000 females for the mosquito species in our study is low compared to the MIR for mosquito species in Vietnam, which is in the range of 0.71–1.3/1000 [22, 24], but is slightly higher than that in previous studies on JEV in...
rural areas of Can Tho province (MIR of 0.05/1000) and in suburban Bangkok (MIR of 0.046/1000) [25, 26]. The low infection rate of JEV in mosquitoes, despite its intensive circulation in pigs, could be explained by the possibility that our RT-PCR assay showed lower sensitivity in mosquito pools compared to serum or CSF, the presence of inhibitors in mosquito homogenate [26], or the existence of direct transmission of JEV between pigs, as suggested by the results of a recent experimental study showing that oro-nasal virus excretion can last 5–6 days in pigs, and that pig-to-pig transmission without mosquitoes can occur [27].

Conclusion
In this study, we provide the first full-genome sequences for JEV strains isolated from humans and mosquitoes in Cambodia, and demonstrate the existence in the country of one complete cycle of JEV by detecting JEV strains in humans, mosquitoes and pigs. Moreover, we demonstrate that JEV circulates between pigs, Cx. tritaeniorhynchus and humans in the vicinity of Phnom Penh. Although all of the Cambodian JEV strains belonged to GI, they clustered in two clades, GI-a and GI-b. Our results provide important information following the recent implementation (March 2016) of the JEV vaccine in the Cambodian National Immunization Programme. All of the commercialized vaccines currently available belong to JEV genotype III, but have been shown to be efficacious against other genotypes and strains, with varying degrees of cross-neutralization [28]. Finally, the detection of different JEV strains in Cambodia belonging to different evolutionary clusters highlights the active circulation of JEV, and in this wild and bred birds play a key role. Such cases have rarely been documented in recent decades and deserve to be examined in greater depths.

METHODS
Specimen collection
The samples used for the detection of JEV were collected through the following three projects: the acute meningoencephalitis (AME) study, the SE Ae study and the companion approach for cross-sectoral collaboration in health risk management in southeast Asia (ComAcross) study (www. onehealthsea.org/comacross). The human samples were from two studies on the aetiology of central nervous system disease that were conducted at the two major paediatric hospitals in the south and north of the country: Kantha Bopha Children’s Hospital in Phnom Penh and Jayavarman VII Hospital in Siem Reap, respectively. The first study (AME) started in July 2010 and ended in December 2013. A total of 1160 paediatric AME cases were enrolled in this study: 930 (80.1 %) patients from Jayavarman VII Hospital in Siem Reap and 229 (19.9 %) patients from Kantha Bopha Hospital in Phnom Penh [11]. The second project (SE Ae) started in July 2014 and is scheduled to end by December 2017. By December 2015, 147 encephalitis cases had been recruited for the study from Kantha Bopha Hospital in Phnom Penh. CSF and blood samples were obtained from all patients during the first 2 days of admission and were tested by PCR and/or serology for a spectrum of known or suspected encephalitis-causing viruses (n=21), bacteria (n=12), parasites (n=2) and fungi (n=1). All samples were collected from patients based on the medical judgement of individual clinicians as part of the patient care management process. Specimens were kept at 4 °C after sampling and transported daily to the laboratory at the Institut Pasteur du Cambodge (IPC). Upon arrival in the laboratory, the specimens were immediately processed and frozen at −80 °C until further analysis [11].

Pig and mosquito samples were collected within the framework of the SE Ae and ComAcross projects. The sampling was conducted in two farms, situated in a peri-urban and a rural area, respectively. The peri-urban farm was located in Ta Khmau, in the southern suburbs 10 km from Phnom Penh [23]. This peri-urban area was characterized by both high human density and a mix of urban landscape and rural landscape with cultivated fields. The rural farm was situated in Kandal province, 40 km south of Phnom Penh. In 2014, 2 cohorts of 15 pigs were consecutively raised and monitored in the peri-urban farm, with blood samples collected every 8 to 11 days from April to July 2014 for the first cohort, and from September 2014 to January 2015 for the second cohort [23]. In 2015, 2 other cohorts of 15 pigs were concomitantly raised and monitored in both farms, with blood samples collected every 8 to 11 days from July to October 2015. Blood samples were collected from the jugular vein using plain tubes with a Vacuette system. We monitored the pigs between 2 and 6 months of age, i.e. after the waning of maternal antibodies and before they were sent to the slaughterhouse.

Mosquito surveillance was conducted in 2014 at the peri-urban pig farm concomitantly with the pig monitoring. Mosquitoes were captured with a homemade CDC light trap on the night preceding the blood sampling of the pigs (i.e. every 8 to 11 days from April to July 2014 and from September 2014 to January 2015). During the collection periods, one trap was installed in the pig farm, in an empty pen between two pens occupied by the pigs included in the study. The trap was setup between 5 and 7 pm, which is the reported optimal feeding time for Culex species [29]. The trapped mosquitoes were collected between 6 and 7 am the following day. All mosquitoes were sorted from other insects, kept in the tubes, and then stored in liquid nitrogen before transportation to IPC. All samples were then identified based on the mosquito’s morphological identification keys for southeast Asian countries [30–32]. Captured mosquitoes were collected and separated by their week of collection, genera, species and sex (male or female). After identification, all mosquitoes were returned to the deep freezer (−80 °C) for long-term preservation prior to virus testing.

Molecular diagnostic
RNA extraction
Genomic RNA was extracted from the biological specimens, including blood samples and CSF from human patients,
swine blood and mosquito pools. Viral RNA was extracted from 140 µl of human and swine sera using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Before extraction, mosquitoes were freeze-killed, pooled according to species, sex and week of collection, with a maximum of 10 mosquitoes per pool, and placed in a vial containing 500 µl of sterile PBS with 10 % foetal calf serum and 15–20 ceramic beads. The mixture was homogenized using a MagNA Lyser instrument (Roche, Mannheim, Germany) at 6500 r.p.m. for 50 s. The homogenates were clarified by centrifugation and used for RNA extraction using the Direct-Zol RNA MiniPrep (Zymo Research Corp., USA) according to the manufacturer’s instructions.

**Real-time RT-PCR**

A specific real-time RT-PCR assay was used for the detection of JEV RNA, as described elsewhere [33]. Briefly, 5 µl of RNA was reverse-transcribed and amplified using the SuperScript III Platinum One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA) in a mixture of 10 µl of 2× reaction mix with ROX, 10 µM forward primer (5'-AGAACGGGAAGAYAACCATGACTAA-3'), 10 µM reverse primer (5'-CCGCCGTTCACCATATTGAT-3'), 10 µM of JEV-specific probe (FAM- ACCAGGAGGGCCGG-BHQ1) and 1 µl of SuperScript III RT/Platinum Taq mix. Molecular-grade water was added to the reaction to obtain a final volume of 20 µl. Thermocycling was conducted as follows: reverse transcription at 50 °C for 30 min, hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 20 s.

**Conventional PCR**

All samples deemed positive by real-time RT-PCR screening were subjected to conventional RT-PCR testing, as described previously [34]. Briefly, the RT-PCR was carried out using a JEV-specific sense primer (JE-NS3-1S; 5'-AGAGCGGGGAAAAAGGTCAAT-3') and an anti-sense primer (JE-NS3-4R; 5'-TTTACGCTTTTCTACAGT-3') targeting the NS3 gene with the SuperScript III One-Step RT-PCR system (Thermo Fisher Scientific Inc, Waltham, MA, USA). The RT-PCR was performed at 53 °C for 30 min and 94 °C for 2 min; 40 cycles at 94 °C for 15 s, 53 °C for 30 s and 68 °C for 90 s; and 1 cycle at 68 °C for 5 min. The product was visualized on a 1.5 % agarose gel. DNA sequencing was performed in a commercial facility with an ABI 3730XL analyser (96 capillary type) using the ABI Prism BigDye Terminator Cycle Sequencing kit according to the manufacturer’s instructions (Macrogen, Seoul, South Korea).

**Virus isolation**

The isolation of JEV was performed on all real-time RT-PCR-positive samples using a mosquito cell line (clone C6/36 of Aedes Albopictus cells). Briefly, each acute serum was diluted 1:120 with L15 Leibovitz medium (Sigma Aldrich, Steinheim, Germany) in which 2 % of foetal calf serum was added. Diluted sera were inoculated into 12-well plates containing 100 % confluent C6/36 cells and then incubated for 7 days at 28 °C. The cells were harvested, and JEV infection was confirmed by an immunofluorescence assay using JEV serotype-specific monoclonal antibodies as described previously [35].

**Full-genome sequencing**

The total RNA was extracted from original biological samples or cell culture suspensions using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized from 3 µl of RNA with 2 µl of each nucleotide triphosphate (Eurobio, Courtaboeuf, France), 10 pmol of each JEV primer (Table S1) and 7.5 µl of sterilized distilled water. The mixture was incubated at 70 °C for 5 min and then stored on ice for 2 min. Each tube was then incubated with 1 µl of SuperScript II reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA) and 0.5 µl of RNasin (Promega, Madison, WI, USA) in a final volume of 20 µl for 50 min at 42 °C, and then at 95 °C for 5 min and 4 °C for 1 min.

Five µl of cDNA was then amplified by sequencing PCR using 10 pmol of paired primers for each JEV fragment (Table S1), 1 µl of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA), 10 nmol of each nucleotide triphosphate and 10× Taq buffer for 5 µl, in 50 µl final volume with H2O. The cycling conditions included initial denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. The PCR amplicons were sequenced using the Sanger method at a commercial facility (Macrogen, Seoul, South Korea) as described above.

**Phylogenetic analysis**

Sequences generated from the PCR products from each strain were analysed and assembled using the CLC Main Workbench 5.5 package (CLC bio, Aarhus, Denmark). The JEV reference strains consisted of 36 full-genome sequences used for full-genome, partial NS3 and partial prM sequence analysis and 102 envelope gene sequences available in GenBank and belonging to 5 genotypes: genotypes I to V. Multiple sequence alignment of the Cambodian strains, with reference strains available from GenBank, was conducted with Muscle [36], as available in the Seaview version 4.2.5 package [37]. Phylogenetic analyses were performed using the maximum likelihood method with the general time-reversible model suggested by Jmodeltest [38], and available in MEGA 5.2 [39] with 1000 bootstrap resampling.

**Funding information**

The AME study was supported by the Li Ka Shing Foundation-University of the Oxford Global Health Programme 2007–2010, PathoQuest (convention 04–12), and the Institut de Microbiologie and Maladies Infectieuses (IMMI n°201103). The SEAe project is funded by Avesan Sud and Fondation Total. The ComAcrross project is supported by the European Union (EuropeAid, INNOVATE contract 315–047). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Acknowledgements
The authors thank the patients and parents who participated in the acute meningitis-encephalitis (AME) and southeast Asia encephalitis (SEAE) studies. We thank Dr Marc Lecuit and Dr Paul Newton, principal investigators of the on-going SEAE study, and Magali Herrant, coordinator of the SEAE project (www.seaeproject.org). We thank Dr Aurelie Binot, coordinator of the ComAcross project (www.onehealthsea.org/comacross).

Conflicts of interest
Philipe Buchy is currently an employee of GSK Vaccines but this position has no link with the work presented here. The authors declare that there are no conflicts of interest.

Ethical statement
The AME and the SEAE studies were approved by the Cambodian National Ethics Committee for Human Research (AME, approval #107NECHR/2009; SEAE approval, #156NECHR/2013). All human samples were collected after obtaining informed consent from the patient’s parents or guardians. The animal sampling study was carried out under an animal care permit from the National Animal Health and Production Research Institute (NAHPRl, former National Veterinary Research Institute – NaVRI). Regarding animal welfare, we followed the principles of the World Organization for Animal Health (OIE), chapter 7.8, ‘Use of animals in research and education’.

References


---

**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.